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Journal of Cellular Biochemistry 113:3466–3475 (2012)

Journal of Cellular Biochemistry

Statins Modulate Transcriptional Activity of Heme-Oxygenase-1 Promoter in NIH 3T3 Cells

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ABSTRACT

Statins, inhibitors of HMG CoA reductase, have pleiotropic effects independent of their capacity to lower cholesterol. Heme-oxygenase-1 (HO-1) plays an important role as an anti-oxidant and anti-inflammatory enzyme. In the present study, we used NIH 3T3 cells which express HO-1 to investigate the molecular mechanisms of HO-1 induction by statins. Simvastatin or fluvastatin induced a significant increase in HO-1 protein expression and mRNA levels. Both statins stimulated activity of a mouse HO-1 promoter (−1,287 to +73 bp)/luciferase reporter gene, 3.25 ± 0.23 (Mean ± S.E.M., n = 15, $P < 0.001$, *t*-test) and 3.13 ± 0.33 (Mean ± S.E.M., n = 6, $P < 0.001$, *t*-test), respectively. This effect was more pronounced in the short proximal promoter than the full promoter of HO-1. Gel retardation experiments for C/EBP and upstream stimulatory factor (USF) DNA-binding activities using simvastatin- or fluvastatin-treated cells showed significant nuclear protein-DNA complexes which were supershifted with antibodies specific for C/EBP β and δ or USF-1 and USF-2. Point mutations of the proximal HO-1 promoter (−149 to +73 bp) for the myc/max which binds USF or the C/EBP binding sequences showed a reduction in statin-induced reporter activity whereas no role of the distal C/EBP binding elements located at −4 kb was observed. Moreover, overexpression of mutated C/EBP β and USF factor or the siRNA for both factors supported a role of these transcription factors in statin-dependent induction of HO-1, with a clearer effect for C/EBP. J. Cell. Biochem. 113: 3466–3475, 2012.

KEY WORDS: STATINS; HEME-OXYGENASE-1; C/EBP; USF; siRNA

Heme-oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme. It catalyzes heme degradation to iron, carbon monoxide (CO), and biliverdin [Ryter et al., 2006]. HO-1 is the inducible isoform of HO that plays an important role as an anti-oxidant and anti-inflammatory [Naito et al., 2011]. HO-1 contributes to the protection from vascular inflammation in atherosclerosis [Durante, 2012]. In vascular cells, Lee et al. [2004] demonstrated a protective role of HO-1 through inhibition of NFκB and release of nitric oxide. In inflammatory cells, increase in HO-1 expression plays a role in the resolution of inflammation [Vicente et al., 2003] and confers an important anti-inflammatory effect through the expression of the anti-inflammatory cytokine IL-10 [Lee and Chau, 2002]. In addition, a protective role of HO-1 expression and activity was shown in gastrointestinal diseases [Naito et al., 2011]. Recent studies support a protective role of HO-1 in fibroblasts in many pathophysiological situations including ultraviolet irradiation of skin [Raval et al., 2012], cigarette smoking [Baglole et al., 2008], and osteoarthritis and rheumatoid arthritis [Chi et al., 2012].

Statins are competitive inhibitors of 3- hydroxyl-methylglutaryl coenzyme A (HMG CoA) reductase and potent inhibitors of cholesterol synthesis [Alberts et al., 1980]. Their therapeutical role has been attributed not only to their cholesterol lowering capacity but also to their beneficial pleiotropic anti-inflammatory, anti-oxidant, and anti-thrombotic effects. Studies have shown that

Conflicts of interest: None.

May F. Mrad and Charbel A. Mouawad contributed equally to the work.

Grant sponsor: American University of Beirut (MPP and URB); Grant sponsor: Lebanese National Council for Scientific Research; Grant number: 04-09-07.

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Manuscript Received: 16 April 2012; Manuscript Accepted: 1 June 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 June 2012

DOI 10.1002/jcb.24223 • © 2012 Wiley Periodicals, Inc.

these effects could be in part due to prenylation of proteins [Liao, 2005]. Statins, by blocking the conversion of HMG CoA into mevalonate, decrease the level of isoprenoids [Buhaescu and Izzedine, 2007] and reduce prenylation of proteins [Sebti, 2005]. The prenylation of proteins such as the small G-proteins Ras, Rho, and Rac is essential for their translocation to the cell membrane and therefore their activity [Liao and Laufs, 2005; Zhou et al., 2011]. Statins were described to block prenylation of RhoA and enhance the activity of endothelial NO synthase [Laufs and Liao, 2000; Rikitake and Liao, 2005] and cyclooxygenase-2 [Degraeve et al., 2001].

Statins modulate the activity of many transcription factors essential for gene expression. They were shown to increase the kruppel-2 like factor in endothelial cells [Parmar et al., 2005; Sen-Banerjee et al., 2005] or macrophages [Tuomisto et al., 2008], GATA-6 in vascular smooth muscle cells [Wada et al., 2008] and Keap-1/Nrf-2 which controls the expression of different protective genes in response to oxidative stress [Habeos et al., 2008]. In vascular cells, cardiac fibroblasts and macrophages, statins were shown to decrease activation of NFκB and AP-1 [Dichtl et al., 2003; Holschermann et al., 2006; Habib et al., 2007; Sundararaj et al., 2008]. Recent reports have shown that HO-1 induction by statins involved AP-1 activation [Chen et al., 2006] and kruppel factor -2 [Ali et al., 2007]. C/EBP are members of the basic-leucine zipper transcription factor superfamily (bZIP) consisting of two subfamilies, one group comprises C/EBP α, β, δ and another consisting of γ, ε, and ζ. C/EBP transcription factors are activated in response to cytokines such IL-6 and INFγ, stress, inflammatory mediators, and hypoxia [Schrem et al., 2004]. Recently, reports showed a role for C/EBP in gene regulation and expression [Tsukada et al., 2011]. Upstream stimulatory factor (USF) are members of the basic-helix loop/helix leucine zipper proteins involved in cell development, immune response, cell proliferation, and cell stress [Atchley and Fitch, 1997].

Since statins have anti-inflammatory and anti-oxidant effects and since HO-1 was shown to play a protective role in cells, we reasoned that the anti-inflammatory and anti-oxidant properties of statins may in part be due to induction of HO-1 expression. Consistent with this hypothesis, we show that two statins, simvastatin, and fluvastatin, activate HO-1 gene transcription in NIH 3T3 murine embryonic fibroblasts cells. We also demonstrate that this process involves the mevalonate pathway and the inhibition of geranylgeranylation. We further show that statin treatment increases HO-1 promoter activity, through the action of USF and C/EBP β and δ transcription factors and that dominant negative (DN) mutants of the two transcription factors or the attenuation of their expression with siRNA significantly decreases the effect of statins on HO-1 expression.

MATERIALS AND METHODS

MATERIALS

Murine fibroblasts NIH 3T3 were obtained from American Type Culture Collection (ATCC; Manassas, VA). Simvastatin, Fluvastatin, GGTI-286, and FTI-297 were from EMD-Calbiochem (San Diego, CA). Mevalonate was from Sigma-Aldrich (St. Louis, MO). γ-^{32}P ATP (6,000 mCi/mmol) was from NEN (NEN Life Science Products, Boston), T4 kinase, Enhanced Chemiluminesence ECL kit and poly (dI/dC) were from General Electric (Piscataway, NJ). Donkey anti-rabbit and anti-mouse IgGs conjugated to horseradish peroxidase were from Jackson Immunolaboratories (West Grove, PA). Stbl2 bacteria and fetal bovine serum were from Invitrogen (Carlsbad, CA). XLe-1 bacteria were from Stratagene (La Jolla, CA). Rabbit polyclonal antibodies anti- USF-1, USF-2, CEBP-α, CEBP-β, and CEBP-δ were from Santa Cruz Biotechnology (Santa Cruz, CA). Restriction enzymes, Tripure, X-tremeGENE siRNA Transfection Reagent, Mini and Maxi plasmid preparation kits and β-Gal reporter assay kit were from Roche (Mannheim, Germany). Luciferase Reporter Assay kit was from Promega (San Luis Obispo, CA). RT-PCR kit and taq polymerase were from Finnzymes (Esposo, Finland). Culture media and chemical reagents were from Cambrex (Rockland, ME). C/EBP β dominant-negative vector was described earlier and USF-1 DN vector was kindly provided by Dr. Charles Vinson [Szentirmay et al., 2003]. pCMV 500 and pCMV 566 empty vectors were used as control for C/EBP β and USF-1, respectively. pCH110–lacZ vector was from General Electric. All HO-1 promoter reporter vectors were described previously [Alam et al., 1994]. siGenome siRNA for USF-1/2, C/EBP α, β, and δ were from Thermo Scientific Dharmacon (Lafayette, CO). All chemicals reagents were of highly pure grade and were from Amresco (Solon, CA) or Biorad (Hercules, CA).

CELL CULTURE AND TREATMENT

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM Hepes (pH 7). To study HO-1 protein expression, NIH 3T3 (3×10^5 per well) cells were seeded in 12-well plates and incubated 24 h later in FBS-free DMEM culture media for 24 h in the presence or absence of 25 μM simvastatin or 10 μM fluvastatin. For gel retardation experiments, cells were seeded to sub-confluence in 150 mm dishes and treated, in serum-free medium, with 25 μM simvastatin or 10 μM fluvastatin for different periods of time. For reporter experiments, cells (5×10^4 per 12-well plate) were transfected and treated with statins 24 h post-transfection unless otherwise indicated. In some experiments, mevalonate was added 30 min prior to the addition of statins.

PROTEIN EXPRESSION AND WESTERN BLOT ANALYSIS

After treatment, cells were lysed on ice with 200 μl of lysis buffer (20 mM Tris-HCl pH 7.5, containing 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF). Total protein content was determined using the Bradford Assay (Bio-Rad) with BSA as standard. HO-1 and β-actin protein expression were analyzed as described previously using 30 μg of total proteins and 1/2,000 polyclonal rabbit anti-mouse HO-1 antibodies [Alcaraz et al., 2000], 1/10,000 of anti-β-actin monoclonal antibody, and 1/2,000 donkey anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase. Signals were developed using enhanced chemiluminescence ECL according to manufacturer's instructions.

REPORTER ASSAY

Different lengths of the HO-1 promoter gene, 0.149, 1.3, 3.7, 4.3, 15 kbp, ΔE1-15 kbp corresponding to the full 15 kbp minus the

enhancer 1 region, and the 0.149 Kbp promoter harboring mutations for the C/EBP or myc/max binding elements were described previously [Alam and Den, 1992; Alam et al., 1994]. Cells were transiently transfected with 4 μg of each HO-1 promoter fusion-luciferase reporter construct and 1 μg of positive control pCH110-LacZ, using the calcium phosphate precipitation, treated with statins as described in the different experimental conditions and lysed in 100 μl β-galactosidase lysis buffer. The luciferase activity was measured in 25 μl of cell lysate using the Promega Luciferase Reporter Assay System, while the β-galactosidase activity was measured in 25 μl of cell extract using Roche β-Gal Chemiluminescent Reporter Assay System. Relative luciferase and galactosidase activities were assessed, according to manufacturer's instructions. Ratio of luciferase to β-galactosidase were established and fold of untreated cells were determined where untreated cells were attributed a value of 1.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Gel retardation experiments were performed for the binding of C/EBP and USF myc/max, using the oligonucleotides HO-1 C/EBP (sense): 5′-GCTGGATGTTGCAACAGCAGC-3′, and HO-1 USF (sense): 5′-GGCCACCACGTGACCCGC-3′. Single stranded oligonecuclotides, at a concentration of 100 pmol/μl, were annealed, phosphorylated with [^{32}P]- γ ATP (6,000 Ci/mmol), and incubated with 5 μg of NIH 3T3 nuclear extract in reaction buffer 20 mM Tris pH 7.9 containing 120 mM KCl, 25 mM $MgCl_2$, 2 mM EDTA, 25% Glycerol, 2 mM DTT, and 1 μg of Poly dI/dC in a final volume of 20 μl for 20 min at 25°C as previously described [Habib et al., 2007]. Cold competitor or mutant double strands DNA were added in excess to designated tubes. In supershift experiments, 2 μg of rabbit polyclonal IgG for CEBP α, β, and δ or USF-1 and USF-2 or unrelated transcription factor antibodies were added 30 min before the addition of the labeled probe. The samples were submitted to eletrophoresis on 6% non-denaturing polyacrylamide gel. The gel was then dried, and radioactive signals were detected using the Storm PhosphorImager (General Electric).

REVERSE TRANSCRIPTASE-PCR (RT-PCR) ANALYSIS OF HO-1 AND β-ACTIN

Sub-confluent NIH 3T3 cells in 60 mm petri dishes were treated with simvastatin or geranylgeranyltransferase inhibitor GGTI-286 for 24 h and total RNA were isolated using Tripure® (Roche). RT-PCR was performed using the Phusion RT-PCR Kit (Finnzymes). Two micrograms of total RNA are transcribed into single-stranded cDNA and PCR is performed using selective primers for HO-1 and β-actin. HO-1 (F): 5′-GAATTC AGCATGCCCCAGGATTTG-3′; HO-1 (R): 5′-TCTAGACTAGCTCAATGT TGAGCAGGA-3′; β-Actin (F): 5′-GTGACGAGGCCCAGAGCAAGAG-3′; β-Actin (R): 5′-AGGGGCCG-GACTCATCGTACTC-3′.

TRANSFECTION OF CELLS WITH DN MUTANT CONSTRUCTS OF USF-1 AND C/EBP β

Sub-confluent NIH3T3 cells in six-well plates at 70% confluency were transfected with 3 μg of control empty expression vectors PCMV 566 or PCMV 500 and their DN USF-1 or C/EBP β mutants, respectively, using EXGen 500 (Fermentas, Glen Burnie, MD). Forty-eight hours post transfection, cells were treated with 25 μM simvastatin for 24 h and lysed for HO-1 protein expression.

TRANSFECTION OF CELLS WITH SMALL INTERFERING RNA OF USF-1 AND 2 AND C/EBP α, β, AND δ

NIH3T3 cells in six-well plates at 50% confluency were washed once with serum and antibiotic-free medium and transfected with 100 nM of small interfering RNA USF 1 and 2 or CEBP α, β, and δ (from Dharmacon) using X-tremeGene siRNA transfection reagent in serum-free medium. Four to six hours post-transfection, the medium was changed. Transfection was repeated once after 24 h. Cells were then treated with 25 μM simvastatin or 10 μM fluvastatin for 24 h and western blotting was performed to detect HO-1, USF-1/2, CEBP β, and β-actin proteins.

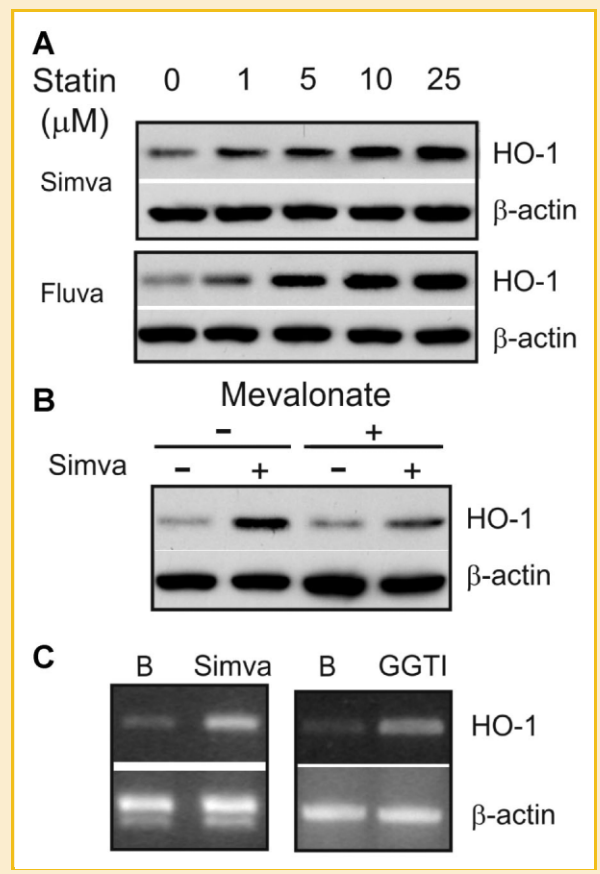

Fig. 1. Effect of statins and GGTI-286 on HO-1 in murine fibroblasts NIH 3T3. A: Dose response of simvastatin or fluvastatin in cells. Cells were incubated for 24 h with increasing concentrations of simvastatin or fluvastatin. Twenty micrograms of total proteins were loaded on a 12% SDS–polyacrylamide gel and blotted for HO-1 and β-actin. Results are representative of three experiments with similar results. B: Effect of mevalonate. 200 μM of mevalonate were added to cells 30 min prior to the addition of 25 μM of simvastatin for 24 h. Data are representative of three experiments with similar results. C: Effect of statins and GGTI-286 on HO-1 mRNA levels. NIH 3T3 cells were incubated with 25 μM of simvastatin or 10 μM of GGTI for 24 h. RNA template was reverse transcribed and then amplified with primers specific for HO-1 and β-actin. The figure is representative of three separate experiments for simvastatin and two separate experiments for GGTI with similar results.

STATISTICAL ANALYSIS

Autoradiograms obtained from western blot analyses were scanned using an Epson 1680 scanner and densitometric analyses were performed using Scion image software. Results of reporter assays were expressed as the mean ± S.E.M. for at least three independent experiments, which were performed in triplicates. An unpaired *t*-test was used for the comparison of two groups, and *P*-values were reported (SigmaPlot, Systat Software Inc., San Jose, CA).

RESULTS

SIMVASTATIN AND FLUVASTATIN INCREASE HO-1 IN NIH 3T3 CELLS

Treatment of NIH 3T3 cells for 24 h with different concentrations of simvastatin or fluvastatin resulted in a significant increase in HO-1 expression. Induction of HO-1 was detectable at 1 and 5 μM and reached a maximal induction at 25 μM for simvastatin and at 10 μM for fluvastatin (1.9 ± 0.12 and 1.8 ± 0.11 fold of control ± S.E.M., n = 10 and n = 6, $P < 0.001$, *t*-test for simvastatin and fluvastatin, respectively) (Fig. 1A). Induction of HO-1 was inhibited by 80% in cells pretreated 30 min with 200 μM of mevalonate prior to the addition of 25 μM simvastatin for 24 h compared to cells treated with simvastatin alone (Fig. 1B). Addition of 25 μM of simvastatin for 24 h to cells increased HO-1 mRNA levels which were detected by RT-PCR (Fig. 1C). We further checked the effect of a selective geranylgeranyl transferase inhibitor on HO-1 expression. 10 μM of GGTI-286 increased the level of mRNA for HO-1 compared to untreated cells (Fig. 1C). FTI-276, a selective farnesyltransferase inhibitor, did not increase the level of HO-1 mRNA (data not shown).

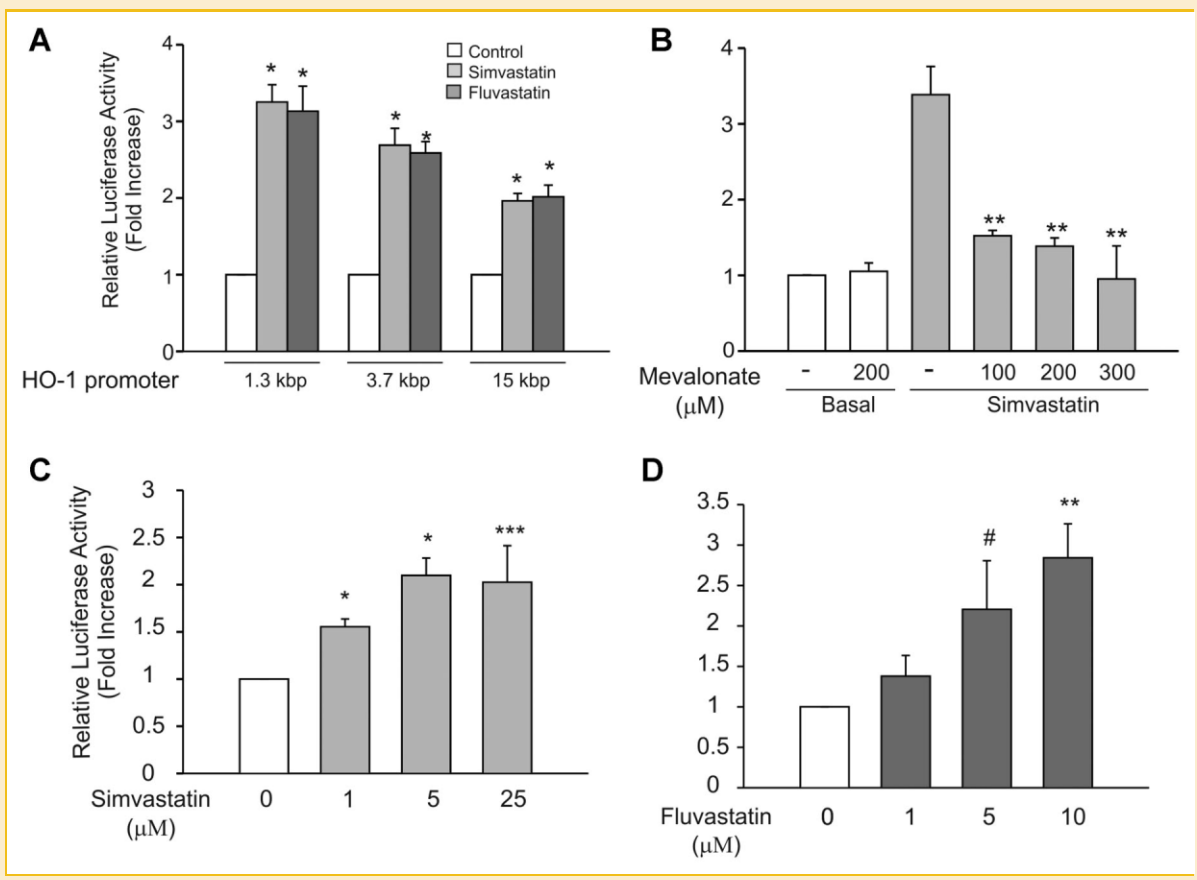

Fig. 2. Effect of statins on HO-1 promoter activity: (A) Effect of statins on HO-1 promoter reporter assay. Cells were transiently transfected with reporter luciferase vectors containing different lengths of the HO-1 promoter and the pCH110-LacZ construct. Twenty-four hours post-transfection, 25 μM simvastatin or 10 μM fluvastatin were added for an additional 24 h incubation period, after which cells were lysed. Firely luciferase activity was normalized to the β-galactosidase activity expressed as fold increase compared to untreated cells to which a value of 1 was attributed. Results are represented as mean ± S.E.M. of n = 17, 11, and 23 for simvastatin treated 1.3, 3.7, and 15 kbp and n = 12, 6, and 16 for fluvastatin treated 1.3, 3.7, and 15 kbp HO-1 gene promoters, respectively. B: Effect of mevalonate on simvastatin-induced HO 1.3 kbp promoter activity in NIH 3T3. Twenty-four hours post-transfection, 100, 200, or 300 μM of mevalonate were added 30 min prior to the addition of 25 μM simvastatin for 24 h. Data are mean ± S.E.M. of three separate experiments. C: Dose response activation of pHO-1 1.3 kbp construct by simvastatin. Twenty-four hours post-transfection with the 1.3 kbp promoter and pCH110-LacZ, 1, 5, and 25 μM simvastatin were added for 24 h. Data are mean ± S.E.M. of three separate experiments. D: Dose response activation of HO-1 1.3 kbp construct by fluvastatin. Twenty-four hours post-transfection with the 1.3 kbp promoter and pCH110-LacZ construct, 1, 5, and 10 μM fluvastatin were added for 24 h. Data are mean ± S.E.M. of three separate experiments. * $P < 0.001$ versus control; ** $P < 0.003$ versus simvastatin; *** $P < 0.01$ versus control, # $P < 0.03$ versus control, Unpaired *t*-test.

SIMVASTATIN AND FLUVASTATIN INCREASE HO-1 PROMOTER ACTIVITY

To investigate whether statins modulate HO-1 promoter activity, we transfected the cells with luciferase reporter gene vectors driven by the murine HO-1 promoter regions of 1.3, 3.7, and 15 kbp [Alam, 1994]. As shown in Figure 2A, both statins increased promoter activity in a statistically significant manner compared to untreated cells. 25 μM of simvastatin increased the 1.3, the 3.7, and the 15 kbp HO-1 promoter-luciferase activity by 3.25 ± 0.23 ($n = 15$, $P < 0.001$, *t*-test), 2.69 ± 0.73 ($n = 11$ $P < 0.001$, *t*-test) and 1.96 ± 0.44 ($n = 21$, $P < 0.001$, *t*-test) compared to untreated cells, respectively. Similarly, 10 μM of fluvastatin increased the 1.3, the 3.7, and the 15 kbp HO-1 promoter luciferase activity by 3.13 ± 0.33 ($n = 6$, $P < 0.001$, *t*-test), 2.59 ± 0.15 ($n = 6$, $P < 0.001$, *t*-test), and 2.1 ± 0.15 ($n = 14$, $P < 0.001$, *t*-test) compared to untreated cells, respectively. Maximal HO-1 promoter/luciferase activity was obtained with the 1.3 kbp fragment. Both statins showed a statistically significant decrease in reporter gene activity with the 15 kbp promoter compared to the 1.3 kbp promoter (40 and 36% inhibition for simvastatin and fluvastatin, respectively) (Fig. 2A). Pretreatment of cells with different concentrations of mevalonate (100–300 μM) for 30 min prior to the addition of statins resulted in a strong inhibition of HO-1 expression by 80% (Fig. 2B). The 1.3 kbp HO-1 promoter activities were increased dose-dependently by simvastatin (Fig. 2C) and fluvastatin (Fig. 2D).

GEL RETARDATION ANALYSES IN RESPONSE TO STATINS

To examine which transcription factor modulates the DNA-binding activity in response to statins in these cells, we performed gel retardation experiments using double stranded (ds) DNA containing sequences corresponding to C/EBP and myc/max binding sites located in the proximal promoter of HO-1. As shown in Figure 3A, simvastatin and fluvastatin induced protein-DNA complex for C/EBP formation after 12 and 24 h of treatment. DNA-protein complexes were detected at 6 h in response to statins (data not shown). The complex formed in response to statins was competed by excess of cold ds-DNA for C/EBP but not for USF or mutant C/EBP (Fig. 3A,B). Antibodies against C/EBP β and δ but not C/EBP α or unrelated antibodies such as anti-USF, caused supershifts in the complexes suggesting the presence of C/EBP β and δ in these complexes (Fig. 3B). Moreover, significant protein-DNA binding was obtained for myc/max containing sequences that bind USF transcription factors, in cells after 12 and 24 h treatment with simvastatin (Fig. 4A) or fluvastatin (Fig. 4B). DNA protein complexes were detected at 6 h (data not shown). This binding was competed with excess cold ds-DNA containing myc/max sequences but

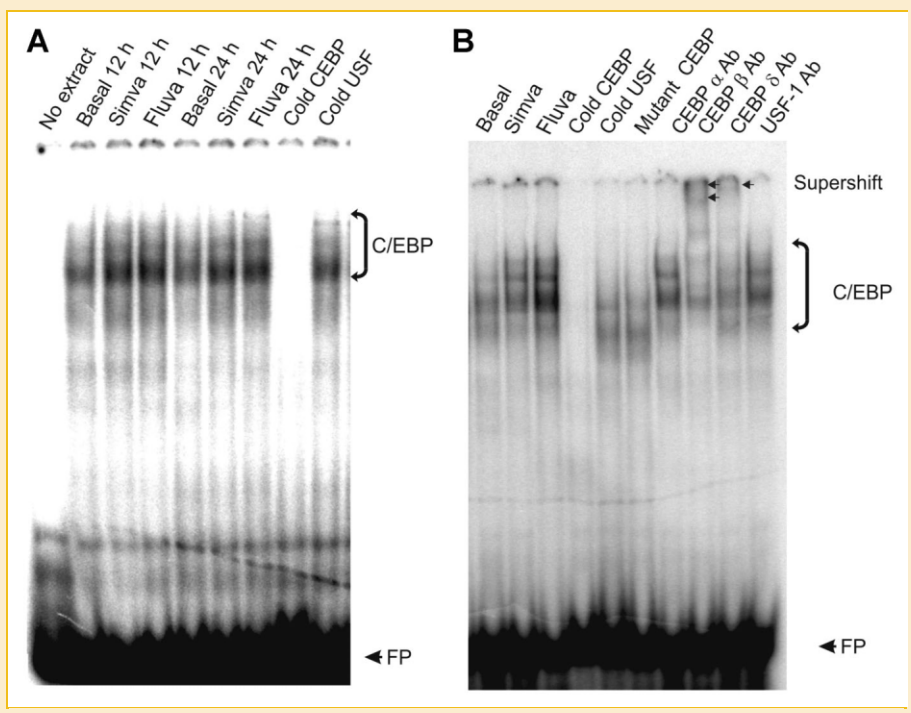

Fig. 3. Electrophoretic mobility of C/EBP-HO-1 DNA complexes in response to statins. A: Cells were treated, in serum free medium, with 25 μM simvastatin or 10 μM fluvastatin for 12 and 24 h. Nuclear extracts were prepared and gel retardation assay was carried out as described in "Materials and Methods" section. In vitro nuclear protein-DNA binding was analyzed using mouse ^{32}P-labeled HO-1 C/EBP probe, and an excess of unlabeled C/EBP oligonucleotide was used as a competitor. Free probe and reaction mixtures were electrophoresed on a 6% polyacrylamide, 0.25% TBE gel. The large arrow indicates the DNA-transcription factor complex. Results are representative of two experiments for simvastatin 12 h and four for 24 h, three experiments for fluvastatin at 12 h, and five for 24 h. B: Supershift analysis of C/EBP-HO-1 DNA complexes. Cells were treated with 25 μM simvastatin or 10 μM fluvastatin for 24 h. Where indicated, polyclonal antibodies specific for CEBPα, CEBPβ, CEBPδ, or USF-1 were added to nuclear protein prior to the addition of the labeled dsDNA and DNA-protein complexes were analyzed as previously described. Arrows indicate the supershifted bands. Results are representative of four and five different experiments for simvastatin and fluvastatin, respectively.

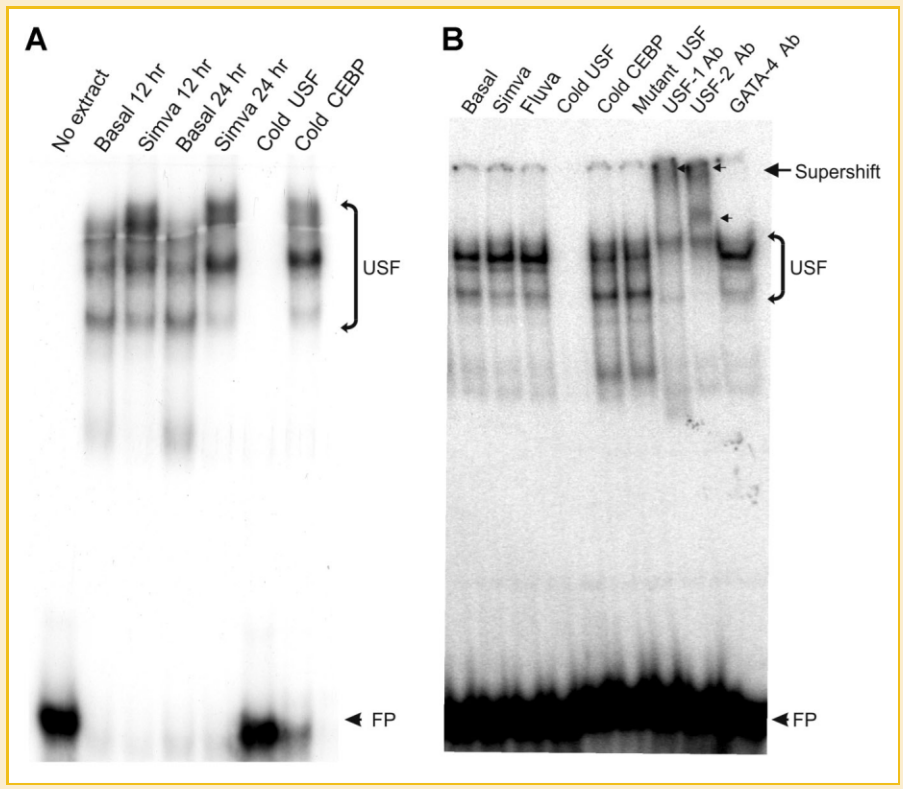

Fig. 4. Electrophoretic mobility of USF–HO-1 DNA complexes in response to statins. A: Cells were treated, in serum free medium, with 25 μM simvastatin for 12 and 24 h. Nuclear protein–DNA binding was analyzed as described in the legend for Figure 3. The large arrow indicates the DNA–transcription factor complex. Results are representative of four different experiments for simvastatin at 12 h and two different experiments for simvastatin at 24 h. B: Supershift analysis of USF–HO-1 DNA complexes. Cells were treated with 25 μM simvastatin or 10 μM fluvastatin for 24 h. Where indicated, polyclonal antibodies specific for USF-1, USF-2, or GATA-4 were added to nuclear protein prior to the addition of the labeled dsDNA and DNA–protein complexes were analyzed as previously described. Arrows indicate the supershifted bands. Results are representative of two different experiments with similar results.

not C/EBP (Fig. 4A) or with mutant USF sequences (Fig. 4B). Pretreatment with both antibodies for USF-1 and USF-2, but not for GATA -4 antibodies, showed a supershift of the DNA complexes (Fig. 4B).

ROLE OF C/EBP AND USF IN HO-1 INDUCTION BY STATINS

Next, we analyzed the effect of mutating the C/EBP or the USF binding sites on promoter activity. For these studies we employed a parent reporter construct containing 149 bp of the HO-1 proximal promoter. Treatment of transfected cells with simvastatin showed a significant reduction, although weak, of the luciferase activity when each of the transcription factor binding sites was mutated (Fig. 5A). Double mutant C/EBP and myc/max showed very low basal activity with no further increase in the presence of statins (data not shown). In order to check any effect of C/EBP binding sequences present in the enhancer region E1 located at approximately −4 kbp upstream of transcription initiation site of mouse HO-1 promoter [Alam, 1994], we compared the promoter activity of the 4.3 kbp-luc HO-1 promoter construct and the 3.7 kbp-luc HO-1 promoter, which does not contain the C/EBP binding elements, in response to statin. Figure 5B shows a similar reporter activity in response to fluvastatin for the two promoter constructs. Moreover, the transfection of cells with the full 15 kbp HO-1 promoter containing a deletion of the enhancer region E1 (ΔE1-kpb) showed no statistically significant difference in HO-1 induction by either simvastatin or fluvastatin in comparison with the full 15 kbp HO-1 promoter (Fig. 5C). These results suggest no role of the C/EBP binding sites located in the distal regions of the HO-1 promoter in response to statins.

To further demonstrate that C/EBP and/or USF were critically required for HO-1 expression induced by statins, we used DN constructs for C/EBP β or USF-1 and checked the effect of statins on HO-1 expression. DN experiments revealed that HO-1 expression was decreased in both cells transfected DN of USF-1 (Fig. 6A) or C/EBP β (Fig. 6B). Control empty vectors, pCMV 566 or 500, for USF-1 and C/EBP β, respectively, did not modify HO-1 expression. When deletion of C/EBP α, β, and δ or C/EBP β alone was performed using specific siRNA, a reduction in HO-1 expression was observed in response to 10 μM fluvastatin (Fig. 6C, upper panel) or 25 μM simvastatin (Fig. 6C, lower panel). This transfection also resulted in a decrease of C/EBP β expression in the presence of C/EBP siRNA (Fig. 6D). For USF-1/2 deletion using siRNA, HO-1 expression was slightly reduced compared to random siRNA (RDM siRNA), although USF expression was reduced (Fig. 6D).

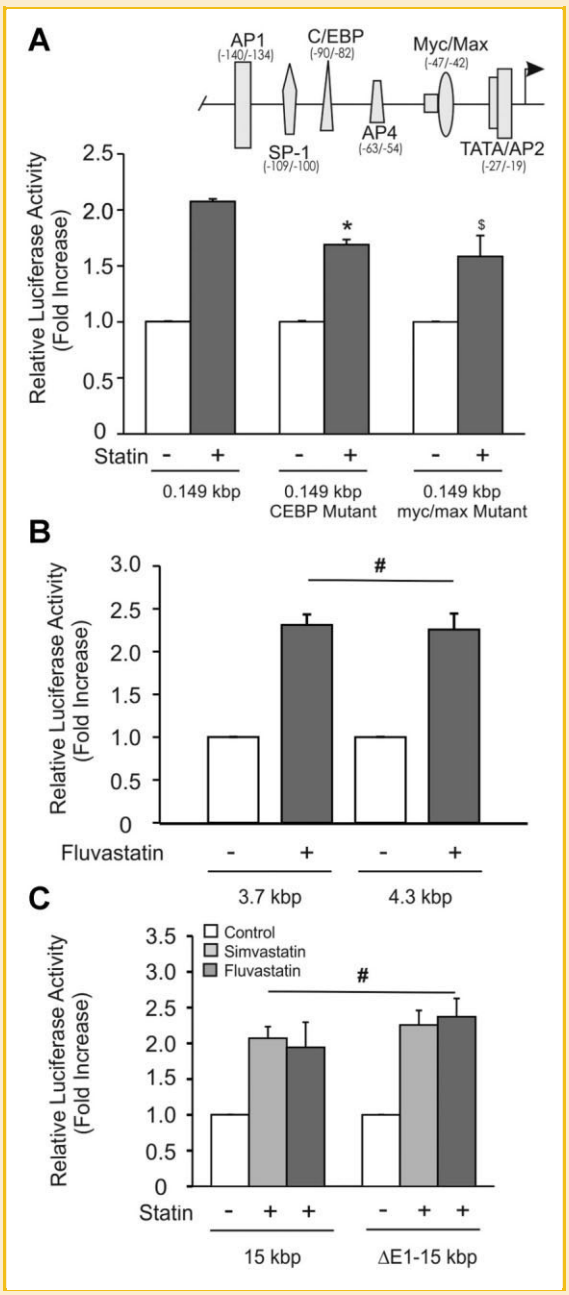

Fig. 5. Role of C/EBP and USF in the proximal region of HO-1 promoter gene in statin-induced HO-1 activity. A: Cells were transiently transfected with the 0.149 kbp region of HO-1 promoter gene or the same construct harboring a mutation in the binding sites for C/EBP or USF along with pCH110-LacZ construct. Twenty-four hours post-transfection, 25 μM simvastatin was added for 6 h. Means ± S.E.M. of four separate experiments are shown. $^{\$}P < 0.01$, $^{*}P < 0.001$ versus 0.149 kbp. Insert illustrates the 0.149 kbp region of the HO-1 gene promoter. B: Cells were transiently transfected with the 3.7 and 4.3 kbp regions of HO-1 promoter gene along with pCH110-LacZ construct. Twenty-four hours post-transfection, 10 μM fluvastatin was added for 12 h. Means ± S.E.M. of four separate experiments are shown ($P < 0.001$ vs. untreated cells, $^{\#}P = 0.818$ for 4.3 kbp vs. 3.7 kbp). C: Cells were transiently transfected with HO-1 distal promoter region 15 kbp and its deletion construct of enhancer 1(ΔE1) at −4 kbp along with pCH110-LacZ construct. Twenty-four hours post-transfection, 25 μM simvastatin or 10 μM fluvastatin were added for 24 h. Means ± S.E.M. of six separate experiments are shown ($P < 0.001$ for statin-treated cells vs. untreated cells, $^{\#}P > 0.35$ for 15 kbp vs. ΔE1-15 kbp).

DISCUSSION

In the present study, we showed that statins induced HO-1 expression in NIH 3T3 fibroblast cell line. Statins were shown to decrease oxidative stress in multiple cells systems and in the context of inflammation and fibrosis, such as macrophages [Tuomisto et al., 2008], osteoblasts [Huang et al., 2012], mesangial cells [Solini et al., 2011], bronchial cells [Schaafsma et al., 2011; Iwata et al., 2012]. HO-1 is induced in response to reactive oxygen species and inflammatory mediators. Increase in HO-1 expression has been shown to protect cells against oxidative stress, DNA damage, and fibrosis and to be anti-inflammatory [Li et al., 2003; Kim et al., 2006; Kie et al., 2008]. These cytoprotective effects against oxidative injury are described in different organs including liver, lung, vessels, and kidney [Durante, 2012]. Studies have shown that CO and biliverdin, products of HO-1 activity, have beneficial protective effects [Paine et al., 2010]. In our study, we demonstrated that one of the antioxidant effects of statins involves induction of HO-1. We also investigated the transcriptional regulation of the HO-1 gene in response to statins in a fibroblast cell line NIH3T3. We have shown that simvastatin and fluvastatin, two structurally different statins, increased HO-1 expression in a mevalonate-dependent manner and involved inhibition of geranylgeranylation.

Fibroblasts play a critical role in the synthesis of extracellular matrix and tissue repair [Tyrrell, 2004; Bartok and Firestein, 2010]. However, activation of these cells in response to tissue injury and oxidative stress results in the synthesis of many pro-oxidant and pro-inflammatory mediators and is associated with excess matrix deposition leading to fibrosis and tissue alteration. Examples of induction of HO-1 were reported in fibroblasts where it contributes to limit cell and tissue damage such as in cigarette smoke-treated fibroblasts or in synovial fibroblasts [Baglole et al., 2008; Chi et al., 2012].

We studied C/EBP and USF involvement in the statin effect on HO-1 expression. Statins increased the activity of HO-1 promoter fragments and the reporter response was observed within the 149 bp fragment of the proximal promoter, a region that includes C/EBP, USF, and AP-1 binding elements [Alam, 1994; Alam et al., 1994]. These transcription factors are key regulators for many genes implicated in cell arrest, anti-proliferative gene responses, immune responses, lipid metabolism, and oxidative stress to UV [Fisher et al., 1992; Corre et al., 2009]. Gel retardation studies showed significant association of C/EBP β and δ to specific binding DNA sequences derived from the proximal promoter region of the HO-1gene. C/EBP β is capable of forming dimers with other transcription factors including CREB, c-Jun, or c-Fos [Lee et al., 1997]. Our results suggest that statins, which are largely described with anti-inflammatory and anti-oxidant effects, increase the activation of C/EBP β and HO-1 expression. Mayer et al. [2007] showed recently that rosuvastatin decreases C/EBP activation by IL-6 in hepatocytes [Mayer et al., 2007]. Although C/EBP β is activated by proinflammatory cytokines such as IL-6, we demonstrated in our system that both simvastatin and fluvastatin increased C/EBP β activation. This activation was important in statin-dependent induction of HO-1 since overexpression of DN of C/EBP b or siRNA of C/EBP b reduced significantly this induction.

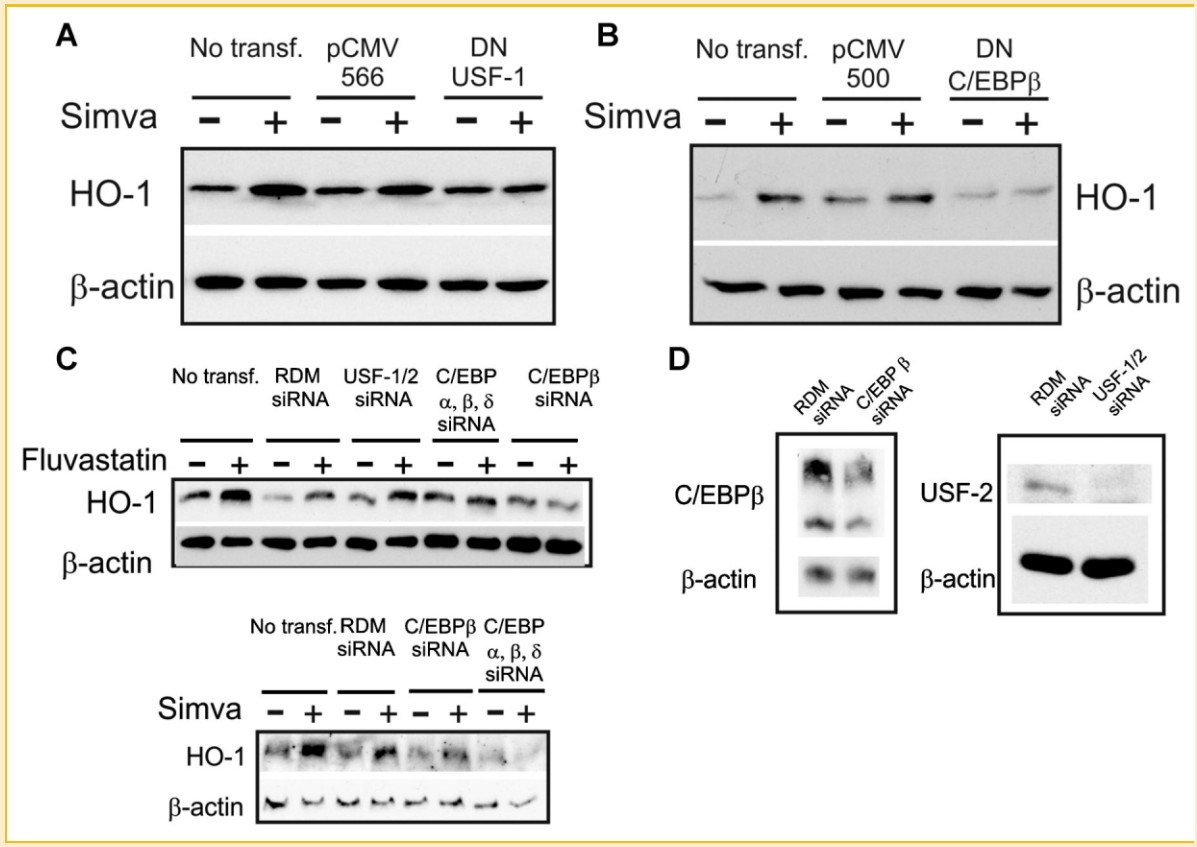

Fig. 6. Role of C/EBP and USF in HO-1 expression. A: Cells were transiently transfected with USF-1 dominant negative vectors or the pCMV 566 empty vector. Twenty-four hours post-transfection, 25 μM simvastatin was added for 24 h. Cells were lysed and western blot analyses were carried out as described in the legend for Figure 1. Results are representative of three experiments with similar results. B: Cells were transfected with C/EBP β dominant negative vector or the pCMV 500 empty vector. Forty-eight hours post-transfection, 25 μM simvastatin was added for 24 h. Cells were lysed and western blot analyses were carried out as described in the legend for Figure 1. Results are representative of two experiments with similar results. C: Cells were treated 24 h after a double transfection with siRNA USF 1 and 2 or CEBP α, β, and δ with 10 μM fluvastatin for 24 h. RDM represents random siRNA. Cells were then lysed and analyzed by western blotting for HO-1 (C) or for CEBP β or USF-1 (D). Results are representative of two different experiments with similar results.

We also checked the role of other C/EBP motifs present in the enhancer 1 region located at −4 kbp of the HO-1 promoter. The 15 kbp mouse HO-1 promoter gene contains a proximal region of 1.3 kbp rich in binding sites for many transcription factors including the AP-1 family, C/EBP, and USF-1 whereas two enhancer regions are located at ~10 and 4 kbp upstream of the transcription start site. HO-1 activation involves various transcription factors in response to LPS, hypoxia, heavy metal and heme, which includes AP-1, C/EBP, HIF, and Nrf2 [Alam and Den, 1992; Lee et al., 1997; Chen et al., 2006; Alam and Cook, 2007]. We compared the full 15 kbp HO-1 promoter construct deleted of the enhancer region E1 to the full promoter 15 kbp and the activity of the distal promoter containing the enhancer region 1 compared to the 3.7 kb HO-1 promoter and showed no role of the enhancer region E1.

We also demonstrated that statins activate USF-1 and 2. When a dominant-negative mutant of USF-1was overexpressed, we observed a decrease in statin-dependent induction of HO-1, which implies a role of USF-1 in the transcriptional regulation of HO-1 by statins. The results of siRNA for USF were less convincing, probably due to an absence of complete inhibition of the expression of USF-1 and USF-2. Recently, USF-1 and 2 were shown to be involved in the transcription of the human HO-1 promoter by binding to the E-Box motif [Hock et al., 2004]. It has been recently demonstrated that p38 MAP kinase is important in the activation of USF-1 [Corre et al., 2009] and that this activation involves both phosphorylation and acetylation. Since p38 MAP kinase is activated in response to statins and plays a role in HO-1 expression in many cellular systems [Zhang et al., 2002; Carlin et al., 2007], it is possible that USF activation by statins is dependent on p38 MAP kinases. USF transcription factors were shown to be involved in blocking cell growth and proliferation. Using single mutations in each of the motifs for binding C/EBP and USF located at −71 bp or USF at −41 bp in the proximal HO-1 promoter, we described a small but significant reduction in the HO-1 promoter activity. Whether association of different transcription factors is needed for the statin effect will need further investigation. Transcription repressors were also described for HO-1. One of the examples is Bach 1, a MAF related transcriptional repressor which associate with MAF, a member of the basic-leucine zipper factors that comprises NFE2 and Nrf-2. MAF members associate with the antioxidant response elements (ARE) motifs present on the HO-1

promoter and are retained by Bach 1. It has been proposed that oxidative stress inactivates Bach 1 resulting in HO-1 gene expression [Igarashi and Sun, 2006; Hira et al., 2007]. Our results showed a higher increase in luciferase reporter activity in cells transfected with the proximal 1.3 kbp HO-1 promoter gene compared to the −3.7 and 15 kbp suggesting an important role of the proximal promoter and probably an inhibitory role of regulatory regions situated beyond this area. Since Nrf-2 is more involved in the distal enhancer regions E1 and E2, it is unlikely that Nrf-2 is involved in statin-dependent activation of HO-1 [Pugazhenthi et al., 2007].

In summary, we showed that the protective effect of statins involves upregulation of HO-1 and investigated the molecular mechanisms involved. This modulation could present one of the beneficial pleiotropic effect of statins. The C/EBP β and USF transcription factors are part of the molecular mechanisms of anti-oxidant properties of statins. We found that statin-induced HO-1 expression in NIH 3T3 depends the activation of the HO-1 promoter with a major effect on the proximal fragment of the HO-1 promoter. The binding of the C/EBP β, δ, and USF proteins, which played a role in HO-1 expression, is evidenced by the use of DN mutants and siRNA of C/EBP and USF. The beneficial cytoprotective effects of statins against cell injury and oxidative stress is related to the increase in expression of HO-1 and involved the activation of some transcription factors such as USF and C/EBP.

AUTHOR CONTRIBUTIONS

M.F.M., C.A.M., performed research and wrote the paper, M.A.A performed research; A.A.E., J.A., and A.H. designed research and wrote the paper.

ACKNOWLEDGMENTS

This work was supported by grants from the American University of Beirut (MPP and URB) and the LNCSR (grant #04-09-07). We are grateful to Dr. Charles Vinson (Laboratory of metabolism, National Cancer Institute, Rockville) for providing the DN mutant of USF-1 and to Dr. Georges Nemer (department of Biochemistry, American university of Beirut) for his advice in setting up gel retardation experiments.

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